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Structure of the asparagine-linked sugar chains of porcine kidney and human urine cerebroside sulfate activator protein

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The specific sugar residues and their linkages in the oligosaccharides from pig kidney and human urine cerebroside sulfate activator proteins (saposin B), although previously hypothesized, have been unambiguously characterized. Exhaustive sequential exoglycosidase digestion of the trimethyl-*p*-aminophenyl derivatives, followed by either matrix-assisted laser desorption/ionization and/or mass spectrometry, was used to define the residues and their linkages. The oligosaccharides were enzymatically released from the proteins by treatment with peptidyl-*N*-glycosidase F and separated from the proteins by reversed-phase high-performance liquid chromatography (HPLC). Reducing termini were converted to the trimethyl-*p*-aminophenyl derivative and the samples were further purified by normal-phase HPLC. The derivatized carbohydrates were then treated sequentially with a series of exoglycosidases of defined specificity, and the products of each digestion were examined by mass spectrometry. The pentasaccharides from pig kidney and human urine protein were shown to be of the asparagine-linked complex type composed of mannose-α1–6-mannose-β1–4-*N*-acetylglucosamine-*N*-acetylglucosamine(α1–6-fucose). This highly degraded structure probably represents the final product of intra-lysosomal exoglycosidase digestion. Oligosaccharide sequencing by specific exoglycosidase degradation coupled with mass spectrometry is more rapid than conventional oligosaccharide sequencing. The procedures developed will be useful for sequencing other oligosaccharides including those from other members of the lipid-binding protein class to which cerebroside sulfate activator belongs. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: cerebroside sulfate activator protein; saposin B; oligosaccharide structure; electrospray mass spectrometry; matrix-assisted laser desorption/ionization; exoglycosidase digestion; trimethyl-*p*-aminophenyl derivatization

INTRODUCTION†

Cerebroside sulfate activator, also referred to as the GM1-activator,[1] sphingolipid activator protein 1 (SAP-1),[2] the non-specific activator protein[3] and saposin B,[4] is a small, heat-stable protein involved in the catabolism of cerebroside sulfate (sulfatide) and several other glycosphingolipids.[5,6] In this and other publications from our group, the protein is referred to as cerebroside sulfate activator (CSAct) to reflect the principal activity that is measured. Genetic defects in human CSAct lead to arylsulfatase A-positive (activator deficient) forms of metachromatic leukodystrophy, a progressive, inherited neurodegenerative disease.[7] The function of CSAct is believed to involve the binding and solubilization of certain lipids making them available to soluble hydrolytic enzymes. It is thought that this protein might also participate in additional enzymatic activities and facilitate the transport of lipids between membranes.[8]

CSAct isolated from human liver,[9] cultured fibroblasts,[10] kidney[11,12] and urine[13] and pig kidney (PKA)[14,15] is glycosylated at Asn_{21}.[11–15] Molecular mass analyses have indicated that the PKA material contains three predominant glycoforms, the penta-, di- and monosaccharide adducts (5CHO, 2CHO and 1CHO glycoforms, respectively), while the HUA material contains two predominant glycoforms (5CHO and 1CHO glycoforms).[13,16] More highly glycosylated forms of this protein have been isolated from livers of individuals with other lysosomal

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† Abbreviations: CSAct, cerebroside sulfate activator protein; C_8, octyl hydrocarbon chain; dHex, deoxyhexose; ESIMS, electrospray ionization mass spectrometry; 5-, 2-, 1- and 0-CHO, 5, 2, 1 and 0-oligosaccharide containing; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; $(Hex)_n$, *n* hexose units; $(HexNAc)_n$, *n* *N*-acetylhexose units; HPLC, high-performance liquid chromatography; HUA, human urine CSAct; LC/MS, liquid chromatography/electrospray ionization mass spectrometry; Man, mannose; MS/MS, tandem mass spectrometry; NP, normal-phase; MALDI, matrix-assisted laser desorption/ionization mass spectrometry; P, parent ion; PNGase, peptide *N*-glycosidase; PKA, pig kidney CSAct; RP, reversed-phase; TFA, trifluoroacetic acid; TMAPA, trimethyl-*p*-aminophenylammonium chloride; W/A/F, water–acetonitrile–formic acid (50 : 50 : 0.1, v/v).

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storage disorders, but these do not appear to occur under normal metabolic conditions.[17] Glycosylation at Asn_{21} is apparently essential for normal intracellular functioning of the protein because two different mutations resulting in loss of the *N*-glycosylation site are among the mutations which lead to activator deficient forms of metachromatic leukodystrophy. Two cases of an Asn to Lys substitution (N21K using the CSAct nomenclature, or N215K using the precursor prosaposin nomenclature) at the glycosylation site have been reported.[18,19] This mutation, which results in loss of a highly conserved amino acid residue and abolishes the only *N*-glycosylation site of the protein, causes severe and progressive neurological deterioration. Another form of activator-deficient metachromatic leukodystrophy is caused by a single point mutation (C–T transition) in the open reading frame resulting in a substitution of isoleucine for threonine (T23I using the CSAct nomenclature, or T217I using the precursor prosaposin nomenclature) and loss of the glycosylation recognition site.[20,21] However, purified normal (wild-type) protein that has been enzymatically deglycosylated *in vitro* appears either very similar to or indistinguishable from normal glycosylated protein by a number of criteria. These include the ability to support *in vitro* aryl sulfatase A-catalyzed sulfate hydrolysis from cerebroside sulfate,[12,23] resistance to proteolytic enzymes,[22] the hydrogen–deuterium exchange profile,[23] circular dichroism[23] and the ability to correct CS metabolism in CSAct deficient cells grown in culture (C. B. Fluharty *et al.*, unpublished data). Therefore, it is unclear just how these mutations result in a metabolic deficiency. The differences between wild-type protein and the T23I and N21K mutant proteins have been suggested to involve *in vivo* sequalae of glycosylation, possibly including the role of glycosylation in protein folding and/or loss of the recognition site necessary for correct intracellular trafficking and localization.[22] However, the prosaposin precursor must be adequately processed since function of the other co-encoded saposins appears normal in CSAct defects.

To appreciate fully the role of glycosylation in the biology of this protein, a method is required for the rapid characterization of the glycosyl moiety including definition of the individual sugar residues and their linkages. In this paper such a method is described and applied to CSAct from pig kidney and human urine. This work is part of on-going detailed biophysical investigations which are a necessary prerequisite to understanding the structure, specificity and modes of action of this protein.

EXPERIMENTAL

Materials

TFA (HPLC grade in 1 ml glass ampoules) was purchased from Pierce (Rockford, IL, USA), acetonitrile (HPLC grade) and methanol (Optima grade) from Fischer Scientific (Tustin, CA, USA), *N*-glycosidase F (PNGase F) from Boehringer Mannheim (Indianapolis, IN, USA) and oligosaccharide standards (maltotriose, -pentaose and heptaose), sodium cyanoborohydride and trimethyl-*p*-aminophenylammonium chloride (TMAPA) from Aldrich (Milwaukee, WI, USA). β-*N*-Acetylglucosaminidase (#722S), α1–3, 4-fucosidase (#723S), α1–2-fucosidase (#724S), α1–6-mannosidase (#727S), α1–6-mannosidase (#727S), α1–2,3-mannosidase (#729S) and β-mannosidase (#732S) were obtained from New England Biolabs (Beverly, MA, USA). *N*-Acetylglucosamidase (HEXase I), FUCase I, FUCase II and FUCase III were supplied by Glyco (Novato, CA, USA). The sources of these enzymes and their specificities are presented in Table 1. Quartz-distilled water (>16 mΩ cm^{-1}) was produced in-house and all other reagents and solvents were of analytical grade or better.

CSAct purification

Pig kidney activator (PKA) and human urine activator (HUA) were prepared as described previously.[13,14] The final 25 mM Tris–HCl (pH 7.5) size-exclusion chromatographic eluates were concentrated in an Amicon Diaflo Ultrafilter (YM2 membrane, 3 kDa molecular mass cut-off, under 40 psig nitrogen pressure). The PKA preparation contained about 27 mg protein ml^{-1} and the HUA

Table 1. Listing of the specificities and sources of the exoglycosidases used

Enzyme	Specificity	Source
722S	β-D-*N*-acetylglucosamine	*Xanthomonas manihotis*
723S	α1,3-and α1,4-fucose	*X. manihotis*
724S	α1,2-Fucose	*X. manihotis*
727S	α1,6-Mannose	Cloned from *X. manihotis* and expressed in *E. coli*
729S	α1,2-and α1,3-mannose	Cloned from *X. manihotis* and expressed in *E. coli*
732S	β1,4-Mannose	*Xanthomonas holcicola*
HEXase I	β1,2-, 1,3-, 1,4-and 1,6-*N*-acetylglucosamine	Cloned from *Diplococcus pneumoniae* and expressed in *E. coli*
FUCase I	α1,6-Fucose	Cloned from *Flavobacterium meningospeticum* and expressed in *E. coli*
FUCase II	α1,2-Fucose	Cloned from *X. manihotis* and expressed in *E. coli*
FUCase III	α1,3-and α1,4-fucose	Cloned from *X. manihotis* and expressed in *E. coli*

preparations contained between 3 and 7.5 mg protein ml^{-1} (Lowry protein assay using BSA as standard).

Enzymatic deglycosylation of CSAct and collection of released oligosaccharide

Deglycosylation was carried out as described previously.[16] Native protein was treated with *N*-glycosidase F and the resulting components were separated by extended C_8 RP-HPLC. The non-retained fraction eluting in the first 10 min was collected and the acetonitrile was removed in a vacuum centrifuge. The remaining solvent was then removed by lyophilization and the dried fractions were stored at −80 °C. Material recovered from the deglycosylation of 20.5 μmol of PKA and 2 μmol of HUA was available for these experiments. Previous work has shown that the PNGase treatment cleaves only the longer sugar residues from the protein and leaves the 2CHO and 1CHO glycoforms untouched.[16] Calculations of the amounts of protein-derived carbohydrates used in the various experiments were based on the molar amounts of protein used for deglycosylation, and assuming a 50% recovery of carbohydrate based on the approximate proportion of pentasaccharide-containing protein in the PKA and HUA starting materials.[16]

Derivatization of reducing termini

The dried RP-HPLC flow-through fractions, containing the PNGase-released protein carbohydrates, were resuspended in water (typically 100–1000 μl), centrifuged (3000 *g*, 5 min) and aliquots of the supernatants were transferred to clean glass screw-capped (PTFE-lined) tubes. Chemical derivatization was accomplished as described previously[24] by treating the aqueous solutions with a 10-fold molar excess of TMAPA over carbohydrate in reaction volumes between 100 and 1500 μl of water at 90 °C for 5 min. Following this, an equal amount of additional TMAPA was added with sodium borohydride (10-fold molar excess over available carbohydrate) and glacial acetic acid (1 μl per μl of reaction mixture) and the solution was mixed and heated at 90 °C for an additional 60 min.

Purification of the TMAPA-derivatized oligosaccharides

The dried TMAPA and exoglycosidase reaction mixtures were redissolved in water and then diluted with two volumes of acetonitrile prior to chromatography. Alternatively, the original TMAPA reaction mixtures were chromatographed directly. No significant differences in the fidelity of chromatography between the two modes of sample injection were apparent although retention times were reduced by injection of aqueous sample volumes that were >25% of the per minute column flow-rate. The solutions were subjected to normal-phase partition chromatography (NP-HPLC) by injection on to an amino-bonded silica resin (Keystone Scientific Carbohydrate column, 300 × 4.6 mm i.d., 5 μm particle size, 100 Å pore size) equilibrated in acetonitrile–water and eluted (1 ml min^{-1}) with equilibration solvent for 5 min followed by a linearly increasing gradient of water over the following 50 min to a final solvent composition of 99% water. The acetonitrile–water mixtures used for column equilibration varied between 50 : 50 to 90 : 10 giving elution gradients of 0.98–1.78% min^{-1}. The column effluent was monitored at 215, 254 and 280 nm and the collected 1 ml fractions were dried in a vacuum centrifuge. The dried fractions were redissolved in water–acetonitrile (1 : 1) and aliquots were removed for examination by mass spectrometry and exoglycosidase digestion.

Exoglycosidase digestions

HPLC-purified TMAPA derivatized samples in water–acetonitrile (1 : 1) were dried in a vaccuum centrifuge and redissolved in exoglycosidase buffer (1 : 100 aqueous dilution of the buffer supplied with each enzyme or recommended by the manufacturer, 5 μl) to which was added exoglycosidase (1 μl of the supplied solutions). Samples were incubated typically for 1 h or overnight at 37 °C, following which they were dried in a vacuum centrifuge. They were then either directly analyzed by ESI MS or further purified by NP-HPLC.

Electrospray mass spectrometry

A Perkin-Elmer SCIEX (Thornhill, Ontario, Canada) API III triple-quadrupole mass spectrometer fitted with an Ion Spray™ source was tuned, calibrated and operated in the positive ion mode as described previously.[25] Samples (aliquots of HPLC fractions in water–acetonitrile (1 : 1) and dried exoglycosidase digestion mixtures) were diluted or redissolved in water–acetonitrile–formic (W/A/F, 50 : 50 : 0.1, v/v) and injected (10–20 μl per injection) into a stream of the same solvent entering the ion source (10 μl min^{-1}). Spectra were collected by scanning typically from *m/z* 300 to 1500 with a 0.3 Da step size and a scan speed of about 6 s with an orifice voltage of 90 V. To induce source fragmentation, some spectra were collected at high orifice voltages (150 and 200 V). Positive fragment ion spectra of Q1 pre-selected parent ions were produced by scanning Q3 from *m/z* 50 to 1500 (0.3 Da step size, 1 ms dwell time, 5.09 s per scan, orifice voltage 90 V, argon collision gas thickness instrumental setting (CGT) of 170, $R_0 - R_2$ offset of 30 V). Averaging of all the scans accrued from each sample injection was achieved with the MacSpec computer program (version 3.3, Perkin-Elmer SCIEX).

Matrix-assisted laser desorption/ionization mass spectrometry

Sample solutions (aliquots of HPLC fractions in water–acetonitrile (1 : 1) and exoglycosidase digestion mixtures) were diluted in W/A/F and 1 μl aliquots were co-spotted with an equal volume of 2,5-dihydroxybenzoic acid matrix (10 mg ml^{-1} in acetonitrile–water–TFA (70 : 30 : 0.1, v/v) and spectra were recorded with a reflector time-of-flight instrument (PerSeptive Biosystems Voyager RP) used in the linear mode with external calibration from bovine insulin.

Molecular mass calculations of the TMAPA derivatives

These were made from the monoisotopic (average) residue masses of acetylhexosamine ($C_8H_{15}NO_6$), hexose ($C_8H_{15}NO_6$) and deoxyhexose ($C_8H_{12}O_5$) of 203.08 (203.19), 162.05 (162.14) and 146.06 (146.14) Da, respectively, with the addition of the mass of two protons (2.01 (2.02)) and TMAPA ($C_9H_{15}N_2$, 151.12 (151.22)).

RESULTS

The TMAPA derivatives of the HUA- and PKA-derived materials revealed single peaks of UV absorption during NP-HPLC with maximum intensity in the 254 nm profile (Fig. 1).

MALDI spectra of the 254 nm absorption peak from the PKA preparation revealed a strong signal at *m/z* 1030.8 and weaker signals at *m/z* 884.8 and 868.9 [Fig. 2(A)]. The ESI spectra (collected with a standard orifice voltage setting of 90 V) of the same PKA preparation was essentially identical with a strong signal at *m/z* 1029.7 Da and weaker signals at *m/z* 883.6 and 867.4 [Fig. 2(B)]. The signal at *m/z* 1029.7 corresponds to the TMAPA derivative of a $(HexNAc)_2(Hex)_2dHex$-containing sugar (calculated monoisotopic molecular mass 1029.5 Da), while the signals at *m/z* 883.6 and 867.4 correspond to the TMAPA derivatives of $(HexNAc)_2(Hex)_2$- and $(HexNAc)_2HexdHex$-containing sugars, respectively (calculated monoisotopic molecular masses of 883.4 and 867.4 Da, respectively). The spectra of this sample also revealed the variable occurrence of three other weaker signals, not or barely evident in Fig. 2(A) and (B), at *m/z* 972.7 which corresponds to the TMAPA derivative of a $(HexNAc)_2HexdHex$-containing sugar (calculated monoisotopic molecular mass 972.4 Da), and ions at *m/z* 918 and 810 which remain unassigned. The ESI spectra of the NP-HPLC-purified HUA sugar preparation also revealed a strong signal at *m/z* 1029.7 which is assigned as above [Fig. 2(C)]. This signal was always accompanied by a weak signal at *m/z* 1191.7 which is assigned as arising from the TMAPA derivative

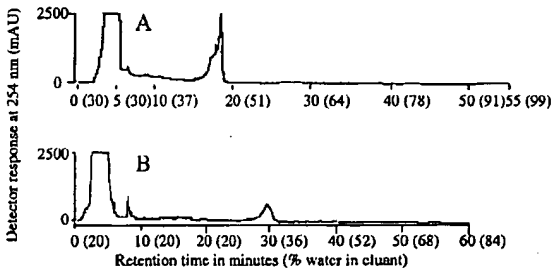

Figure 1. NP-HPLC 254 nm elution profiles of TMAPA-treated PNGase-released carbohydrate from pig kidney (A) and human urine (B) CSAct. Aliquots (250 µl) of the pig kidney TMAPA reaction mixture was injected directly on to the HPLC column. ESIMS of aliquots of the collected fractions showed that the peak eluting at 16–19 min contained the sugars of interest. The human urine TMAPA reaction mixture (800 µl) was diluted with acetonitrile (600 µl) and 250 µl aliquots of this solution were injected. ESIMS on aliquots of the collected fractions showed that the peak eluting at 27–31 min contained the sugars of interest.

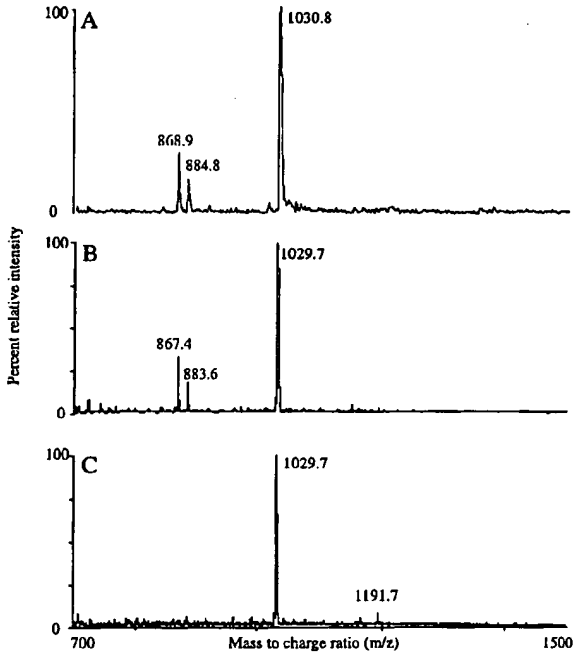

Figure 2. MALDI and ESI mass spectra of TMPA-derivatized, NP-HPLC-purified, carbohydrate derived from PNGase-treated pig kidney CSAct (A and B, respectively), and an ESI mass spectrum of material from human urine CSAct (C).

of a $(HexNAc)_2(Hex)_3dHex$-containing sugar (calculated monoisotopic molecular mass 1191.6 Da).

MS/MS of the TMAPA derivatives of all the PKA-derived sugars showed a 15 Da loss (P − 15 ion) from the respective parent ions (P ion) in addition to a number of fragment ions [Fig. 3(A), (B) and (C)]. The 867.5 [Fig. 3(A)] and 1029.5 [Fig. 3(C)] Da sugars showed losses of 146 (dHex) and 162 Da (Hex) from the P ions, followed by sequential losses of additional 162 and 203 Da (HexNAc). The 883.4 Da sugar [Fig. 3(B)] revealed a series of fragments ions between *m/z* 630 and 690 which are unexplained, in addition to sequential losses from the P ion of 324 Da (two Hex units) and 203 Da (HexNAc). In the lower *m/z* region the spectra of all three sugars showed abundant common fragment ions at *m/z* 366 (unassigned) and 149, and a series of less abundant fragment ions at *m/z* 220.1, 203.9, 191.0, 167.9 and 137.9 Da.

In an attempt to learn more about the structure of the P − 15 and *m/z* 149 fragment ions, ESI spectra were measured at high orifice voltage. With an orifice voltage of 150 V, the ESI spectrum of the PKA-derivatized sugars revealed the presence of both the P (100%) and P − 15 (50%) ions, in addition to a strong signal at *m/z* 149 (55%). MS/MS on the P − 15 ion revealed no significant fragment ions above *m/z* 210, but a strong fragment ion at *m/z* 149 (100%) in addition to a number of other low-abundance fragment ions including ions at *m/z* 203.9, 191.0, 167.9 and 137.9, which were all between 15–25% of the abundance of the *m/z* 149 ion. MS/MS on the *m/z* 149 ion revealed fragment ions at *m/z* 133.1 (10%), 119.9 (50%), 106.1 (65%), 104.0 (40%) and 77 (100%).

The first cycle of exoglycosidase digestions showed that only the enzymes specific for terminal α1,6-mannose

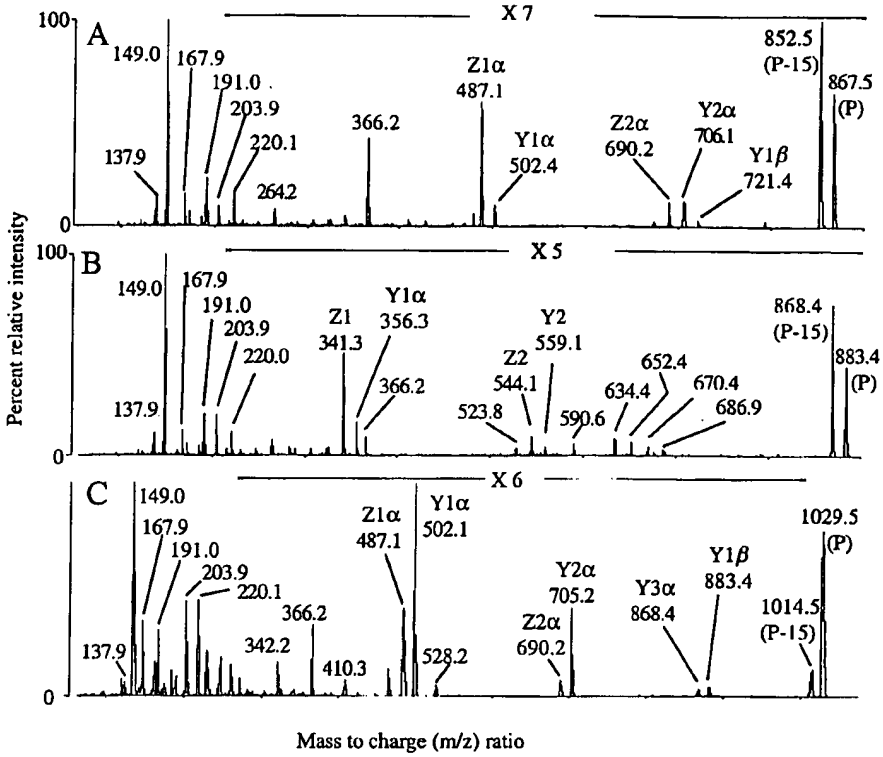

Figure 3. Tandem mass spectra of the TMAPA derivatives of the 867.5 (A), 883.4 (B) and 1029.5 (C) Da pig kidney CSAct-derived carbohydrates. P denotes parent ion. Fragment ion-type assignments are from the nomenclature of Domon and Costello.[37] In all cases, including in the spectra of the linear maltotriose, -pentaose and -heptaose standards, the labeled Z ions were 15 Da lighter than the corresponding Y ions.

(727S) and terminal α1,6-fucose (FUCase I) were capable of using the PKA- and HUA-derived materials as a substrate (Fig. 4). These enzymes reduced the mass of the substrate by 162.0 and 145.9 Da, respectively, consistent with the loss of single mannose and fucose residues and thus identifying the terminal residues and their linkages. Subsequent cycles of exoglycosidase digestion of the PKA-derived substrate showed further sequential losses of 162.1 and 203.1 Da on treatment with enzymes specific for β1,4-mannose (732S) and β1,2-, 1,3-, 1,4-and 1,6-*N*-acetylglucosamine (Hexase I), respectively. The results of sequential exoglycosidase digestion of the PKA- and HUA-derived sugars are summarized in Table 2. The masses of the smallest digestion products (502 Da) are consistent with the TMAPA derivative of a HexNAcdHex-containing molecule (calculated monoisotopic mass of 502.3 Da). Thus in both sugars the fucose residue must be attached to the terminal (reducing) residue. Taken together, the exoglycosidase digestion data prove that the principal glycoform in both the PKA and HUA derived materials consists of mannose α1–6-mannose-β1–4-*N*-acetylglucosamine-*N*-acetylglucosamine (α1–6-fucose), the structure and sites of exoglycosidase cleavage of which are shown in Fig. 5.

DISCUSSION

Two different methods have been used for characterizing the TMAPA-derivatized sugars released from pig kidney and human urine CSAct protein by *N*-glycosidase F treatment. Sequential exoglycosidase treatment coupled with MS identification of the digestion products has been used to identify unequivocably the sugar residues and their linkages. The reliability of sugar and linkage identification with sequential exoglycosidase treatment depends on the exquisite specificity of the enzymes. The tandem mass spectra of the derivatized sugars are reported to provide reference spectra of these molecules for future comparison with the spectra of other sugars of suspected similar structures for their fast and convenient tentative identification. The specific sugar residues and their linkages in the PKA and HUA carbohydrates, although previously hypothesized, have now been unambiguously characterized.

The sugars identified in this work agree with the predictions made from ESIMS molecular mass measurements of the natural glycoforms of the native proteins.[13,16] In this earlier work the 5CHO glycoform of PKA was assigned as $(HexNAc)_2(Hex)_2dHex$ and the 4CHO glycoforms were assigned as $(HexNAc)_2HexdHex$ and $(HexNAc)_2(Hex)_2$. Similar agreement exists between the earlier report on HUA and the new data except that the 4CHO glycoforms previously assigned were not detected in this work, probably because of their low abundance.

Using a combination of methylation analysis, glycosidase digestion and lectin affinity chromatography, Yamashita *et al.*[17] reported a comparative study of the hydrazinolytically released sugar chains on CSAct purified from normal human liver and GM1 gangliosidosis (type 1)

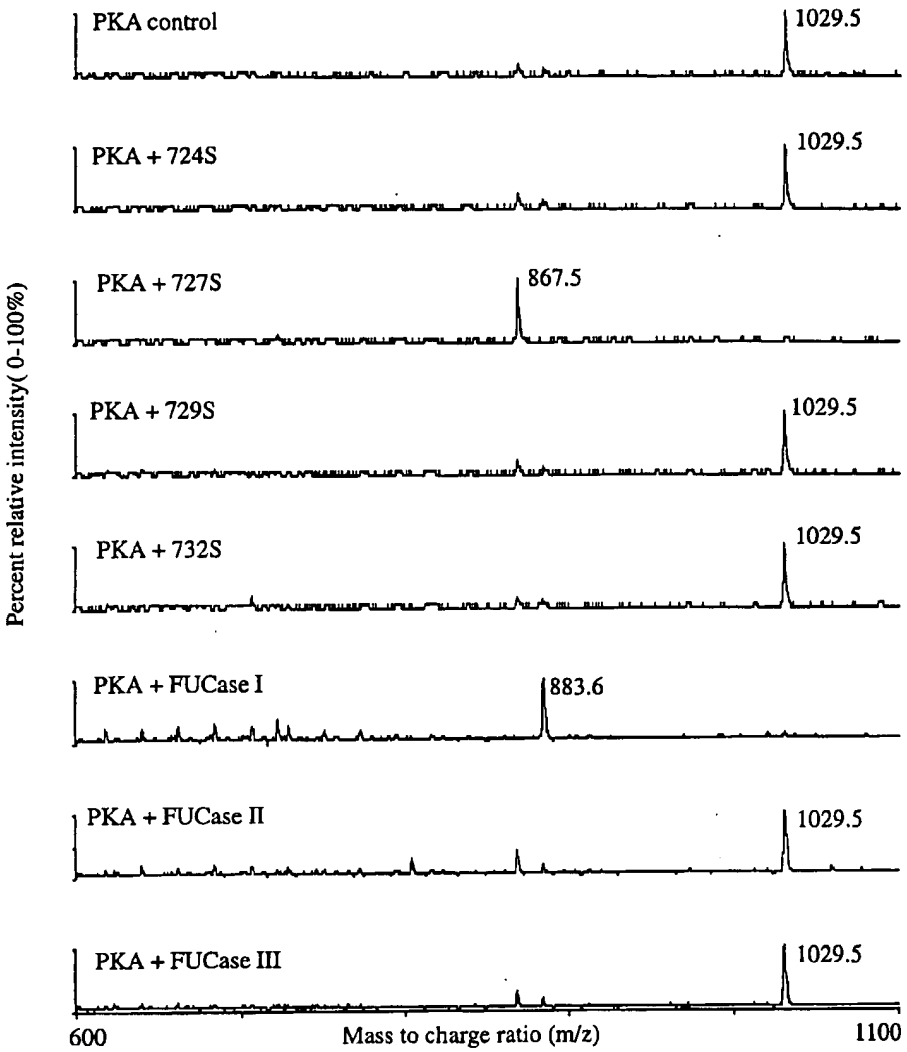

Figure 4. ESI mass spectra of the TMAPA-derivatized oligosaccharides from pig kidney CSAct without treatment (top spectrum) and following 24 h incubation with the indicated enzymes.

liver. Their results showed that the material from normal liver contains a similar mixture of glycoforms as that reported here for HUA. In normal liver the 5CHO glycoform ($Man_2GlcNAc_2Fuc$) was the most abundant component, accounting for 35–40% of the preparation, and a 4CHO glycoform ($Man_2GlcNAc_2$) was the next most abundant, with 1- (GlcNAc), 2- ($GlcNAc_2$), 4- ($ManGlcNAc_2Fuc$), and 6-CHO ($Man_3GlcNAc_2Fuc$) glycoforms each accounting for about 10% of the sample. Two other 5- and 3-CHO glycoforms ($Man_3GlcNAc_2$ and $ManGlcNAc_2$, respectively) accounted for the remaining 5% of the sample. It is clear that the material isolated by Yamashita *et al.*[17] from normal liver and the material used in the present study from pig kidney and human urine does not contain highly mannosylated glycans typical of most glycoproteins. Similar protein purification procedures were used in the present work and by Yamashita *et al.*,[17] and both used a heat denaturing step after tissue homogenization which would minimize any subsequent enzymatic degradation of the glycan chains. Further, following the heat treatment step, only a small proportion of the pig kidney[14] and human urine[13] protein is retained by concanavalin A resin, suggesting that the CSAct from these sources does not contain significant quantities of high-mannose glycoforms. The small size of the glycans attached to the protein isolated from pig kidney and normal human liver and urine is therefore attributed to lysosomal degradation of the complete glycans[17] that are attached to the protein in the rough endoplasmic reticulum and Golgi[26] before relocation to the lysosome and final processing. The inference from these data is that once it has been transported to the lysosome, the biological activity of the protein is not dependent on the presence of high-mannose glycosylation. This conclusion is supported by the observations, previously alluded to, that glycosylated and deglycosylated protein have similar metabolic and physical properties.

The observations by Yamashita *et al.*[17] that generalized gangliosidosis results in liver accumulation of more complex mono-, bi-, tri- and tetra-antennary sugar chains

Table 2. Summary of exoglycosidase digestion of TMAPA-derivatized pig kidney and human urine CSAct-derived sugars[a]

Substrate *m/z*	Exoglycosidase	Product ion *m/z* (% relative intensity[c])	Mass loss (Da)	Inferred terminal residues and linkages
Pig				
1029.7	722S	1029.7	None	
	724S	1029.5	None	
	727S	867.5	162.2	α1–6-Mannose
	729S	1029.5	None	
	732S[b]	1029.5	None	
		705.4 (15–40%)	324.3	
	HEXase I	1029.6	None	
	FUCase I	883.5	146.1	α1–6-Fucose
	FUCase II	1029.6	None	
	FUCase III	1029.7	None	
867.4	722S	867.4	None	
	724S	867.4	None	
	727S	867.4	None	
	729S	867.4	None	
	732S[b]	867.4	None	
		705.4	162.0	β1–4-mannose
	FUCase I	721.4	146.0	α1–6-fucose
705.4	HEXase I	502.3	203.1	*N*-Acetylglucosamine
Human				
1029.4	722S	1029.7	None	
	724S	1029.4	None	
	727S	867.4	162.0	α1–6-Mannose
	729S	1029.4	None	
	732S	1029.4	None	
	HEXase I	1029.7	None	
	FUCase I	883.4	147.0	α1–6-Fucose
	FUCase III	1029.5	None	
867.5	724S	867.5	None	
	727S	867.5	None	
	729S	867.5	None	
	732S	705.2	162.3	β1–4-Mannose
	HEXase I	867.5	None	
705.4	724S	705.4	None	
	727S	705.4	None	
	729S	705.4	None	
	732S	705.4	None	
	HEXase I	502.0	203.4	*N*-Acetylglucosamine

[a] Reaction mixtures were sampled at 1 and 24 h and in some cases after longer incubation times. This tabulation represents data from the 24-h time points which showed complete disappearance of substrate for all samples which registered the formation of a new ion, except incubations labelled [b] which showed residual substrate and product ions in the indicated ratio.
[c] 100% unless stated otherwise.

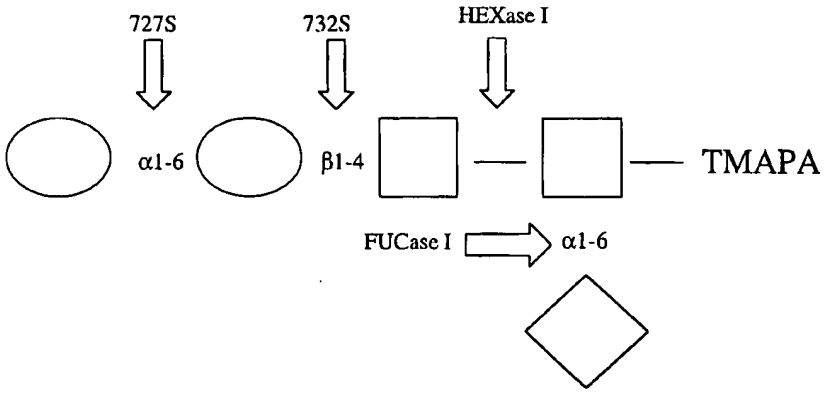

Figure 5. Schematic representation of the structure of the sugar derived from pig kidney and human urine CSAct showing the defined linkages and the site of cleavage of the exoglycosidases. Oval = mannose; square = *N*-acetylglucosamine; diamond = fucose; TMAPA = trimethyl *p*-aminophenylammonium chloride derivative.

containing sialylated and non-sialylated *N*-acetyllactosamine groups supports the contention that more highly glycosylated forms of the protein are transported to the lysosome. Apparently these forms are only detected under pathological situations when the lysosomal degradation of the glycans is overwhelmed. These more complex glycans were not detected in the HUA reported here. As mentioned previously,[16] it remains to be determined if the urine protein from patients with elevated CSAct accumulation is also more highly glycosylated.

The introduction of a chromophore or fluorophore via reducing terminal derivatization, after liberation from the protein, is increasingly used as a method to increase sensitivity of detection of sugars during purification. Judicious selection of the derivatizing group permits the simultaneous incorporation of a constitutive charge or a group with high proton affinity to assist detection by mass spectrometry. Harvey *et al.*[27] have reviewed the available derivatizing groups. As reported previously,[28,29] the TMAPA derivatives gave strong ion currents in both MALDI and ESIMS. The preference for ESI spectra in this study was because of the greater accuracy of *m/z* assignment, but MALDI time-of-flight instruments with delayed ion extraction would provide *m/z* measurements with 10–20 ppm accuracy.[30–32] The MALDI and ESI spectra of the TMAPA derivatives purified by NP-HPLC were not complicated with the presence of alkali metal cationized signals. Such signals are characteristic of the MALDI spectra of other derivatized glycans,[27] and the ESI spectra of TMAPA derivatized maltopentaose purified by water–acetonitrile elution from an amino column.[28] The amount of material required for the collection of MALDI spectra was significantly less (<10%) than that required for collection of ESI spectra using the instruments available for this work. However, at progressively lower *m/z* values, as after two or three cycles of exoglycosidase treatment, the high background from the MALDI matrix complicated data interpretation.

Sample desalting is usually necessary for ESI although less so for MALDI. Dialysis, e.g. by drop deposition on floating membranes (drop dialysis), which has been a convenient method for desalting DNA,[33] proteins[34] and glycans[35] on a microtized scale, was not an option for the purification of the small sugars in this study. The spectra of TMAPA-maltopentaose purified on an amino column by elution with water–acetonitrile are dominated by mono- and disodiated molecular ions.[28] In the work reported here, NP-HPLC worked satisfactorily for the removal of inorganic salts from the original sugar preparation and following chemical derivatization and exoglycosidase treatment, and the resulting ESI and MALDI spectra contained no significant traces of alkali metal-containing ions. Removal of excess quantities of inorganic salts is necessary for the collection of spectra on the Perkin-Elmer SCIEX ESI instrument, although tolerance to inorganic salts in newer ion source designs would reduce the need for some of the chromatography. Although not used here, size-exclusion chromatography could produce the same effect, and graphitized carbon HPLC columns have been shown to be useful for the separation of larger oligosaccharides.[36]

Mass spectrometry alone cannot be used to identify sugars and their linkages because of redundancy in sugar masses and the fact that bond types cannot be unambiguously assigned by MS/MS fragmentation patterns. However, the fingerprint inherent in the MS/MS fragmentation pattern can be used for structural characterization provided that there exists a reference database of spectra of fully characterized sugars. In this regard the tandem mass spectra of the CSAct-derived sugars reported here may be useful because these sugars represent the core of the so-called complex asparagine-linked oligosaccharides. Complex, hybrid and high mannose comprise the three major types of asparagine-linked oligosaccharide.[26]

The tandem mass spectra of the TMAPA-derivatized sugars were surprizingly different, underscoring the difficulty imposed by their interpretation from first principles. Presumably the fragment ions are of the X and particularly Y and Z types of Domon and Costello[37] which arise by charge retention on the reducing terminus, accentuated here because of the quaternary nitrogen in the TMAPA group. These three ion types arise by cross-ring sugar cleavage (X), cleavage on the non-reducing side of the linking oxygen (Y) and cleavage on the reducing side of the linking oxygen (Z). All spectra, including those of the linear TMAPA-maltotriose, -pentaose and -heptaose standards (data not reported), showed strong $[P-15]^+$ ions and strong signals at *m/z* 149. These ions appear to be unique features of the tandem mass spectra of the TMAPA derivatives of sugars. Okamoto and co-workers reported the presence of these ions in ESIMS/MS[28] and post-source decay MALDI-MS[29] of TMAPA-derivatized maltopentaose. The $[P-15]^+$ fragment ions presumably result from loss of a methyl radical, although this explanation invokes the formation of a radical cation which is a rare event under the low-energy collisionally induced dissociation conditions used here. The *m/z* 149 fragment ion was formed at high orifice voltages and following MS/MS of the P – 15 parent. Taken together with the data on the fragment ions produced following MS/MS of *m/z* 149, we assign this ion as being derived from the TMAPA group plus C-1 of the terminal sugar residue ($[CH_2{=}NHC_6H_4N(CH_3)_2]^+$). The tandem mass spectra of the PKA-derived sugars also had a common fragment ion at *m/z* 366. This ion was not evident in the tandem mass spectra of the linear maltose standards, but the structure of this ion remains unassigned.

The tandem mass spectra of TMAPA-derivatized linear maltose standards were relatively uncomplicated and showed the formation of abundant Y-type ions, each accompanied by an ion of similar abundance 15 Da lighter which at the moment are tentatively assigned as the corresponding Z ions. Assignment of corresponding Y and Z ions in the tandem mass spectra of the PKA-derived sugars gave reasonable explanations for the majority of the fragment ions observed, the most notable unassigned ions being the *m/z* 366 fragment ion already referred to above and a series of ions between *m/z* 630 and 690 in the spectrum of the presumably linear 883.4 Da sugar [Fig. 3(B)]. The abundance of fragment ions in these spectra underscores their usefulness for comparative unknown identification.

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